Glial cell functions in CNS homeostasis and local immune regulation Zur

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Glial cell functions in CNS homeostasis and local immune regulation

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LIST OF ABBREVIATIONS

APC	Antigen-presenting cell
BBB	Blood-brain barrier
B7-DC	Programmed cell death 1 ligand 2 (PD-L2)
B7-H1	B7-homologue 1, also known as PD-L1
CCR2	C-C chemokine receptor type 2
CNS	Central nervous system
CTLA-4	Cytotoxic T lymphocyte antigen-4
CX3CL1	Fractalkine
CX3CR1	CX3C chemokine receptor 1, also known as fractalkine receptor
DC	Dendritic cells
DTA	Diphtheria toxin-A
EAE	Experimental autoimmune encephalomyelitis
fALS	Familial amyotrophic lateral sclerosis
GB	Glioblastoma
GFAP	Glial fibrillary acidic protein
GFP	Green fluorescent protein
HLA	Human leukocyte antigen
ICOS	Inducible costimulator
IFN	Interferon
IL	Interleukin
MBP	Myelin basic protein
MHC	Major histocompatibility complex
MS	Multiple sclerosis
MOG	Myelin oligodendrocyte glycoprotein
NSE	Neuron-specific enolase
PBMC	Peripheral blood mononuclear cell
PD-1	Programmed death-1
PLP	Proteolipid protein
RFP	Red fluorescent protein
ROS/RNS	Reactive oxygen and nitrogen species
SOD1	Superoxide dismutase 1
TGFß1	Transforming growth factor β1
T_H	T helper cell
TNF-α	Tumor necrosis factor-α
Treg	Regulatory T cells

SUMMARY

Immune responses within the central nervous system (CNS), welcome or undesired, need to be tightly controlled to protect the CNS tissue from irreparable damage. One of our major interests during the last years was to study the immunoregulatory properties of infiltrating antigen-presenting cells (APCs) and resident glial cells in controlling T cell effector functions within the CNS in the context of glioblastoma (GB) and multiple sclerosis (MS). We focused on B7:CD28 co-stimulatory pathways, given that interrupting the signaling events are attractive strategies to either enhance tumor immunogenicity or control autoimmune inflammation.

GB is a highly aggressive brain cancer that is known for its ability to evade eradication by the immune system. The GB microenvironment actively suppresses effective anti-tumor responses by multiple mechanisms, and we hypothesized that the expression of immune inhibitory B7 family molecules might be one of them (*study I and II*). We could show particularly that high levels of PD-L1 were detectable in all human GB tissue samples examined but not in normal brain regions adjacent to tumor tissue. Alloreactive co-culture assays pointed to a strong inhibitory function of GB-associated PD-L1 on T-cell activation. Our results suggested that PD-L1:PD1 interactions were involved in GB-mediated immune suppression and thus represented a promising therapeutic target. These findings have been confirmed by others and clinical trials evaluating blockers of PD-L1 and its receptor PD-1 in GB patients are underway.

In contrast to anti-GB therapies, enhancing local immunosuppressive properties within the brain is desirable in autoimmune neuroinflammatory diseases such as MS. Manipulation of PD-L1:PD1 interactions may provide a means to control self-reactive T cell activation and effector functions in the CNS. We have found that PD-L1 expression on human monocytes from the peripheral blood was upregulated by the approved MS drug interferon (IFN)-ß and led to reduced APC functions *in vitro* (*study III*). Moreover, IFN-ß treatment elevated PD-L1 levels in MS patients indicating that its anti-inflammatory effects can involve modulation of PD-L1 expression. Further work demonstrated an inhibitory role for PD-L1 on different monocyte-derived,

CNS-infiltrating APC populations and resident microglia in regulating myelin-reactive T-cell activation in experimental MS models (experimental autoimmune encephalomyelitis, EAE; *study IV*). Taken together, our data demonstrated that B7-H1:PD-1 interactions diminished autoreactive T cell responses and restricted immunopathological damage in the CNS.

We then explored how glial cells contribute to the tissue homeostasis of the healthy CNS beyond APC functions and co-inhibition, concentrating on astrocytes. It was reported that astrocytes could promote neuronal survival in co-culture experiments, however the *in vivo* relevance and molecular mechanisms involved in the adult CNS remained unclear. By conditional, astrocyte-restricted protein synthesis termination, we found that selective paralysis of glial fibrillary acidic protein $(GFAP)^+$ astrocytes *in vivo* led to rapid neuronal cell loss and severe motor deficits that were independent of microglial bystander activation (*study V*). Instead, we identified the redox scavenging capabilities of the astroglial cell pool as a critical factor for maintaining neuronal health in the adult CNS.

The work presented here improves our understanding of immunoregulatory and steady state functions of the CNS glial environment and may aid in the design of therapeutic strategies to limit nervous tissue injury.

This cumulative habilitation thesis is based on the following publications:
* - equal contribution

I. **Schreiner B**, Wischhusen J, Mitsdoerffer M, Schneider D, Bornemann A, Melms A, Tolosa E, Weller M, Wiendl H (2003) Expression of the B7-related molecule ICOSL by human glioma cells in vitro and in vivo. **Glia**. 44(3):296-301

II. Wintterle S*, **Schreiner B***, Mitsdoerffer M, Schneider D, Chen L, Meyermann R, Weller M, Wiendl H (2003) Expression of the B7-related molecule B7-H1 by glioma cells: a potential mechanism of immune paralysis. **Cancer Res**. 63(21):7462-7

III. **Schreiner B***, Mitsdoerffer M*, Kieseier BC, Chen L, Hartung HP, Weller M, Wiendl H (2004) Interferon-beta enhances monocyte and dendritic cell expression of B7-H1 (PDL1), a strong inhibitor of autologous T-cell activation: relevance for the immune modulatory effect in multiple sclerosis. **J Neuroimmunol**. 155(1-2):172-82

IV. **Schreiner B**, Bailey SL, Shin T, Chen L, Miller SD (2008) PD-1 ligands expressed on myeloid-derived APC in the CNS regulate T cell responses in EAE. **Eur J Immunol**. 38(10): 2706-17

V. **Schreiner B**, Romanelli E, Liberski P, Ingold-Heppner B, Sobottka-Brillout B, Hartwig T, Chandrasekar V, Johannssen H, Zeilhofer HU, Aguzzi A, Heppner F, Kerschensteiner M, Becher B (2015) Astrocyte depletion impairs redox homeostasis and triggers neuronal injury in the adult CNS. **Cell Reports**. doi: 10.1016/j.celrep.2015.07.051. [Epub ahead of print] PMID: 26299968

INTRODUCTION

Glial cell types: Spotlight on microglia and astrocytes

The term ‘Nervenkitt’ (i.e. nerve-putty) was first introduced by the pathologist Rudolf Virchow in the 1850s, picturing neuroglia as connective tissue that fills up all interstices among nerve cells and their fibers (reviewed in (*1*)). Although most glial cell types and neurons arise from the same embryonic precursor cell, glial cells differ from neurons morphologically and functionally. They do not have the same membrane properties and do not electrically signal like neurons (*2*). Glial cells are a heterogeneous population (reviewed in (*3*)), and are classified as microglia or macroglia which consist of astrocytes, myelin-forming oligodendrocytes in the CNS and Schwann cells in the peripheral nervous system.

Microglia. Microglia are the resident macrophages of the CNS parenchyma that constitute approximately 5-12% of brain cells depending on the region studied (*4*). In contrast to CNS macrophages that are found in the meninges, choroid plexus and perivascular space, microglia are ontogenically distinct as they originate from primitive, yolk sac-derived myeloid progenitors that migrate to the neural tube during early development (*5*, *6*). Studies using bone marrow chimeric animals indicate that the mature microglia pool is maintained independently of recruitment of bone marrow-derived monocytes from the circulation and expands locally even after brain injuries (*7*-*9*). In addition to more fundamental roles in clearing dead cells and debris, microglia have also been implicated in synaptic remodeling as they can engulf and remove inappropriate synaptic connections (*10*, *11*).

Microglia are activated in many neuroinflammatory and -degenerative diseases, and the outcome of this activation may be beneficial, deleterious or neutral (reviewed in (*12*)). Microglial activation leads to disease-associated gene expression (*13*-*15*) and relatively uniform morphological changes converting from a ramified appearance with fine branching processes to an amoeboid shape. In EAE, a mouse model of MS, recent studies support the concept that infiltrating monocyte-derived macrophages/myeloid cells and resident microglia perform non-overlapping functions in the inflamed CNS (*7*,

15). Yamasaki et al. used a dual reporter system to discriminate between CCR2 $(RFP)^+$ monocyte-derived macrophages and CX3CR1 $(GFP)^+$ microglia cells. They showed that monocyte-derived macrophages were highly inflammatory and phagocytic, initiating demyelination at nodes of Ranvier, whereas microglia appeared relatively inert at disease onset (*15*). Whether this is pathogenetically relevant for MS patients is currently unclear.

Astrocytes. Astrocytes descend from neuroepithelial stem cells and are another group of specialized glial cells that are plentiful in the CNS. Their extended processes form gap junctions with each other, contact synapses and nodes of Ranvier and can also ensheath blood vessels. During development, astrocyte-derived molecules such as glypicans appear to regulate synaptogenesis (*16*). It has been proposed that astrocytes have essential 'housekeeping' functions in the healthy adult CNS, including the maintenance of a viable nervous system environment for neurons and the regulation of synaptic transmission and blood flow (reviewed in (*17, 18*). Much of what we know about astrocyte function today has come from *in vitro* studies using isolated glial cells or slice cultures, where they among others promote growth and survival of neurons (*19*). However, global deletion (knockout) of astrocyte intermediate filament genes such as GFAP and vimentin, resulted in no gross neurological, behavioral, or structural CNS abnormalities *in vivo* (*20, 21*). Similar to the studies of microglia biology, advances in transgenic technologies and imaging techniques have begun to allow us to dissect the *in vivo* role of astrocytes in the nervous system function in more detail.

Astrocytes can, like microglia, respond to pathological stimuli through reactive gliosis (reviewed in (*22*)), which might be help- or harmful in several disorders of the nervous system. For example, in chronic brain lesions of individuals with MS, reactive astrocytes form a glial scar (*23*). Several recent loss-of-function studies in EAE indicate that they thereby can restrict the spread of inflammatory cells within CNS tissue, but on the other hand astrocytes might contribute to attracting leukocytes at earlier disease stages (*24-26*). In the setting of spinal cord injury, the glial scar can act as a barrier to the regeneration of damaged nerve fibers (*27*). Moreover, astrocytes are likely important contributors to neurodegenerative diseases such as familial

amyotrophic lateral sclerosis (fALS) (*28*), and can become cancerous, giving rise to gliomas.

The concept of the CNS 'immune privilege'

The concept of 'immune privilege' of the CNS has a long history (*29, 30*). It is based on the observation that immunogens such as xenografts, bacteria, viruses/adenoviral vectors evade immune recognition relative to other organs, when microinjected into the CNS parenchyma. However, peripheral immunization with the same immunogen is followed by a brisk immune response to the antigen deposited within the CNS. The supposed reason for this exceptional position of the CNS is a survival advantage by protecting delicate post-mitotic neurons from inflammatory damage. In addition, pathogens usually enter the CNS from a peripheral site where they already initiated an immune response in the draining lymph nodes and spleen. The term CNS 'privilege' does not relate to the complete absence of immunological components, but rather the CNS and the immune system are tightly coupled and regulated (*30*).

The cellular basis of CNS 'immune privilege' is amongst others the blood-brain barrier (BBB), which excludes plasma proteins, immune cells and their associated inflammatory molecules from the healthy CNS (*31, 32*). In addition, parenchymal dendritic cells (DCs) delivering antigen to the cervical lymph nodes are lacking in the uninflamed brain, although DCs are present in the meninges and the choroid plexus (*33*). This changes once inflammation is established and the 'immune privilege' is undermined. For example, it has been shown that mostly peripherally derived DCs accumulate in the brains and spinal cords of mice with proteolipid protein (PLP) peptide-induced EAE, where they might prime naïve T cells specific for a different PLP peptide locally (leading to epitope spreading (*34, 35*)). Soluble antigen drainage from the CNS to deep cervical lymph nodes occurs as well, and two very recent reports have just reignited the debate if vessels that fulfill the criteria of a lymphatic system are really absent in the CNS (*36, 37*).

Several studies indicate that the CNS resident cells and their microenvironment have immunosuppressive properties during steady state and can actively participate in shaping the immune response within the inflamed CNS. Neurons can inhibit microglial activation in several ways including both cell contact-dependent receptor-ligand interaction (for example membrane-bound CD200 and its receptor (*38*), TREM2-DAP12 (*39*) and others) and secreted molecules (such as soluble fractalkine and its microglial receptor CX3CR1 (*40*), reviewed in (*41*)). Transforming growth factor β1 (TGF-ß1) is a pleiotropic cytokine with potent immunosuppressive and neurotrophic properties that is expressed at low levels by both neurons and glial cells in the normal CNS, but is upregulated in the inflamed brain (*42, 43*). TGF-β1 has been shown to inhibit microglial and astrocyte activation (*44-46*). Moreover, *in vivo*, microglia (and astrocytes) express relatively low levels of major histocompatibility complex (MHC) class II and other accessory molecules that are required for efficient antigen presentation to T cells (reviewed in (*47*)). In addition, microglia and astrocytes produce factors that might directly regulate T cells within the CNS parenchyma (reviewed in (*48*)): For example, using astrocyte-T cell co-cultures it was reported, that astrocytes can suppress T cell proliferation (partially by prostaglandins (*49*)), induce T cell apoptosis (*50*) or regulatory T cells (Tregs (*51*)). Gimsa et al. proposed astrocyte-induced up-regulation of the inhibitory molecule cytotoxic T lymphocyte antigen-4 (CTLA-4) on activated T cells as another immunosuppressive mechanism within the CNS glial environment (*52*).

The B7 family of co-stimulatory molecules

In order to allow T cells to recognize and respond to an antigen it has to be processed and presented on the surface of an APC in the context of MHC complexes. T cells require additional positive signals from APCs for their activation and survival that is provided by co-stimulatory receptor-ligand pairs (signal 2), including the well-characterized B7:CD28 system (*53*) and inflammatory cytokines (signal 3). The B7:CD28 family members have key roles in regulating T cell activation and tolerance. B7-1 (CD80) and B7-2 (CD86) expressed by APCs have a dual specificity for the stimulatory receptor

CD28 on T cells, and the inhibitory CTLA-4 (CD152) that down-regulates T cell responses (*54*). More recently discovered B7 family members such as ICOSL and PD-L1 (B7-H1) and PD-L2 (B7-DC) are not only expressed on professional APCs (DCs, macrophages, B cells) but also on parenchymal cells within non-lymphoid organs (reviewed in (*55, 56*)).

The ICOSL:ICOS pathway. ICOSL has been detected on the surface of professional APCs, and other non-immune cell types such as fibroblasts, endothelial and some epithelial cells (*57-59*). This expression pattern suggests that the outcome of ICOSL:ICOS interactions during immune responses depends not only on their timing but also on the tissue environment (*55*). ICOS is a CD28 homologue and 'inducible co-stimulator' that is expressed on T cells after stimulation by the T cell receptor (*60*). ICOSL:ICOS interactions are important in promoting effective T helper cell (T_H) and B cell responses. The phenotype of 9 patients with common variable immunodeficiency (CVID) lacking ICOS expression on T cells because of an inherited homozygous deletion in the *ICOS* gene indicates its critical involvement in T cell help to B cells (*61, 62*). Moreover, ICOS-deficient mice have abnormal germinal center formation in the spleen and immunoglobulin class switching is defective (*63, 64*). In the absence of ICOS during the priming phase of EAE, disease severity is greatly increased, but paradoxically blockade of ICOS only during the effector phase, improves clinical scores (*64, 65*). This points to a more complex role of the ICOSL:ICOS pathway in T cell regulation, perhaps shifting the balance towards IL-4/IL-10 and humoral at the expense of IFN-γ-mediated cytotoxic/T_H1 responses (*64*). In addition, stimulating IL-10 production may contribute to the regulation of immunosuppressive Tregs and T cell tolerance (*66, 67*). It has been proposed that ICOSL expression by melanoma cells might drive the activation and expansion of Tregs as a mechanism of immune evasion (*68*), but altogether evidence for the expression of ICOSL on other solid tumors is still sparse.

PD1 and its ligands. Whereas PD-L2 is induced by cytokines mainly on macrophages and DCs (*69, 70*), PD-L1 is expressed more widely by B, T, myeloid cells and DCs as well as non-lymphoid tissues (*71-74*) and a variety of tumors (*75, 76*). The PD-1 receptor is upregulated on $CD4^+$ and $CD8^+$ T

cells, B cells, and monocytes upon activation (*77*). PD-L1 and PD1 expression appear to be associated with poorer outcome in several tumors (*78-81*) and their interactions have an important inhibitory function in controlling inflammatory responses in peripheral tissues. PD-L1:PD1 signaling may participate in functionally inactivating 'exhausted' virus-specific $CD8^+$ T cells during chronic viral infections (*82, 83*). Furthermore, PD-1 deficient mice develop lupus-like autoimmune diseases suggesting that PD-1 is involved in the maintenance of peripheral self-tolerance (*84*).

The immune system is not excluded from the CNS, but both are linked and immune responses tightly regulated to protect the nervous tissue from irreparable injury. One of our major interests during the last years was to study the immunoregulatory properties of infiltrating APCs and resident glial cells in controlling T cell effector functions within the CNS in the context of GB and MS. We focused on the B7:CD28 co-stimulatory pathway, given that its manipulation is an attractive strategy to either enhance tumor immunogenicity or control autoimmune inflammation. Subsequently, we have used conditional gene targeting approaches to better understand how glial cells contribute to the tissue homeostasis of the healthy CNS beyond APC functions and co-inhibition, concentrating on astrocytes.

RESULTS AND DISCUSSION

Expression of B7 checkpoint molecules by glioblastoma

I. **Schreiner B**, Wischhusen J, Mitsdoerffer M, Schneider D, Bornemann A, Melms A, Tolosa E, Weller M, Wiendl H (2003) Expression of the B7-related molecule ICOSL by human glioma cells in vitro and in vivo. **Glia**. 44(3):296-301

II. Wintterle S*, **Schreiner B***, Mitsdoerffer M, Schneider D, Chen L, Meyermann R, Weller M, Wiendl H (2003) Expression of the B7-related molecule B7-H1 by glioma cells: a potential mechanism of immune paralysis. **Cancer Res**. 63(21):7462-7

GB is a highly aggressive brain cancer with poor clinical prognosis despite surgery, radiation and chemotherapy (reviewed in (*85*)). GB are exemplary for their ability to suppress effective antitumor responses and actively exploit some of the same immune inhibitory mechanisms that form the basis of the CNS 'immune privilege' described above (reviewed in (*86, 87*). Given the

failure of conventional treatment in GB, immunotherapeutic strategies to enhance the immunogenicity of GB cells are amongst the most promising. In particular manipulations of signal 2 immune checkpoint molecules, such as the B7 family members, to augment antitumor T-cell responses are being tested (reviewed in (*88*)).

The goal of our first study described here (*study I*) was to analyze in detail the expression of ICOSL in biopsy specimens of patients with GB or anaplastic oligoastrocytoma and human malignant glioma cell lines. 3 out of 4 malignant glioma tissue samples stained positive for ICOSL protein by immunohistochemistry. ICOSL could be detected on more than 90% of tumor cells and positive cells were evenly scattered throughout the specimen. Of note, endothelial cells of blood vessels were also $ICOSL^+$. In contrast, no ICOSL was found in normal brain tissue adjacent to tumor tissue or in one brain biopsy specimen without pathological alterations. In addition, ICOSL protein and mRNA was expressed in 7 of 12 glioma cell lines. Its expression was upregulated by the inflammatory cytokine tumor necrosis factor (TNF)-α but not IFN-γ. Neutralizing ICOSL by blocking antibodies in co-cultures of glioma cells with alloreactive peripheral blood leukocytes or T cell subsets ($CD4^+$ and $CD8^+$) reduced T_H1 and T_H2 cytokine levels *in vitro*. However, ICOSL gene transfer into glioma cells did not alter their immunogenicity as determined by alloreactive co-culture assays.

At this point, we did not continue to investigate the cytokine milieu and presence of $ICOS^+$ Tregs in GB samples in more detail. However, in the light of recent studies suggesting that ICOSL expression by melanoma may drive $ICOS^+$ Tregs in the tumor microenvironment (*68, 89*), it would be interesting to determine the levels of ICOSL in a larger number of GB samples and to correlate its expression with tumor-infiltrating Treg numbers and survival. In addition, it has been reported that the frequency of intracranial Tregs was reduced in GL261 glioma-bearing ICOS-deficient compared to wildtype mice, providing rationale for further investigations (*90*).

Instead we focused in a second study on the B7 homologue PD-L1, which binds the strong negative regulator of T cell activation PD-1 (*study II*). Immunohistochemical analysis of malignant glioma specimens revealed strong PD-L1 immunoreactivity in all 10 samples examined, including 9 GB and 1 mixed glioma specimens. More than 50% of the tumor cells expressed PD-L1 in the GB sections (range from 50 to 90%). In contrast, no PD-L1 was found in brain tissue adjacent to tumor tissue or in a normal brain biopsy specimen. Although lacking B7.1/2 (CD80/86), all 12 glioma cell lines constitutively expressed PD-L1 mRNA and protein. Exposure to IFN-γ strongly enhanced PD-L1 expression (**Figure 1**).

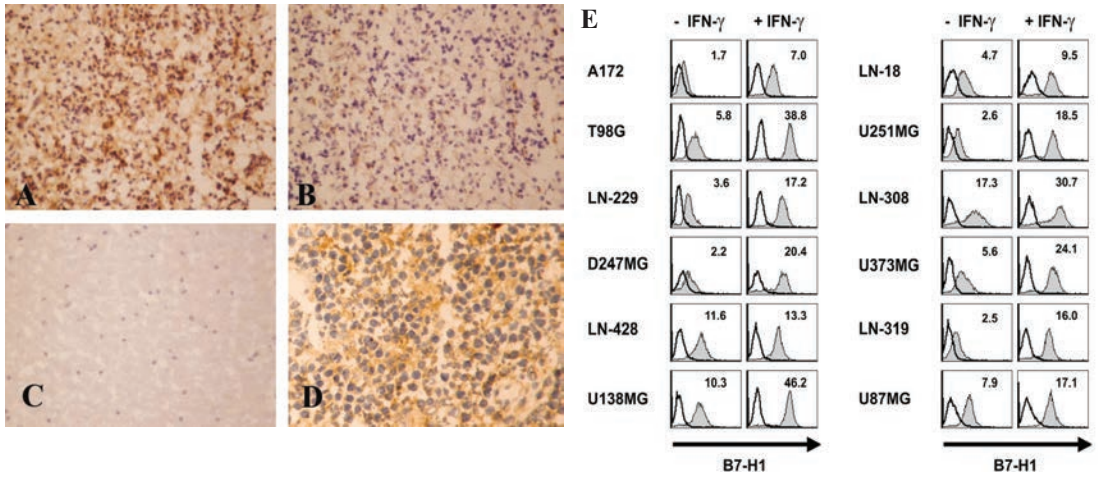

Figure 1. (A-C) Analysis of PD-L1 (B7-H1) expression in brain tumor specimens. Frozen tissue sections of a GB (*A* and *B*), normal brain (*C*), or human thymus (*D*) were immunostained with the PD-L1-specific mAb 5H1 (*A*, *C*, and *D*) or IgG isotype control (*B*). Human thymus served as a positive control (*D*). *A-C*, ×200; *D*, ×400. (E) ■ PD-L1 protein expression at the cell surface of different human glioma cell lines cultured with and without IFN-γ (500 U/ml for 48 hrs) was determined by flow cytometry. The numbers indicate the specific fluorescence index (SFI). □ Isotype control antibody staining. **Adapted from (*91*)**.

To investigate in which ways glioma-associated PD-L1 was functional, we performed coculture experiments of glioma cells with alloreactive $CD4^+$ and $CD8^+$ T cells. The LN-229 glioma cell line used for this set of experiments, exhibited high constitutive levels of human leukocyte antigen (HLA) class I and HLA-DR antigens in addition to PD-L1 but did not express CD80 and CD86. PD-L1 antibody blockade increased $CD4^+$ as well as $CD8^+$ T-cell cytokine production (IFN-γ, interleukin (IL)-2, and IL-10) and levels of the T-cell activation marker CD69, and thereby pointed to a strong inhibitory function of glioma-related PD-L1 on T-cell stimulation.

We concluded that PD-L1 expression may significantly influence the outcome of GB-T cell interactions and may represent an, at that time, novel mechanism by which glioma cells evade immune recognition and destruction. Since then, the main results of this study have been confirmed by several other groups: PD-L1 expression has been demonstrated on established GB cell lines, primary tumors and in addition has been correlated with malignancy tumor grade (*92-95*). In a study by Parsa and colleagues loss of phosphatase and tensin homologue (PTEN) function was linked to PD-L1 expression and immunoresistance in glioma (*96*). Moreover, a considerable percentage of tumor-infiltrating lymphocytes from malignant glioma stained positive for PD-1 (*93*). Immune-based therapies blocking PD-L1 and PD-1 have achieved promising antitumor activity for solid tumors and leukemia patients in the last years (*97-99*), and clinical trials evaluating this approach in GB patients are in development or underway (ClinicalTrials.gov Identifier: NCT02017717 (phase III), NCT01952769 (phase I/II); reviewed in (*86*)).

The role of PD1 ligands on different myeloid cell subsets/microglia in autoimmune CNS inflammation

III. **Schreiner B***, Mitsdoerffer M*, Kieseier BC, Chen L, Hartung HP, Weller M, Wiendl H (2004) Interferon-beta enhances monocyte and dendritic cell expression of B7-H1 (PDL1), a strong inhibitor of autologous T-cell activation: relevance for the immune modulatory effect in multiple sclerosis. **J Neuroimmunol**. 155(1-2):172-82

IV. **Schreiner B**, Bailey SL, Shin T, Chen L, Miller SD (2008) PD-1 ligands expressed on myeloid-derived APC in the CNS regulate T cell responses in EAE. **Eur J Immunol**. 38(10): 2706-17

MS is an autoimmune demyelinating disease of the brain and spinal cord. In most patients, the initial disease course is relapsing-remitting, as inflammatory attacks and neurological deficits are followed by periods of recovery. The disease frequently leads to a continuous and irreversible deterioration in neurologic function termed secondary-progressive MS. The aetiology of MS probably involves both a complex genetic component, in which multiple susceptibility genes are important, and several environmental factors (*100-102*). In MS myeloid APCs are thought to present myelin epitopes to autoreactive T cells in the periphery and CNS (*35, 103*), and thereby propagate an autoimmune inflammatory attack that ultimately overrides local

mechanisms of the CNS 'immune privilege'. As PD-1:PD-L1 interactions are important in inhibiting initial self-reactive T cell activation and expansion but also subsequent T cell effector functions in the target organ (reviewed in (*104*)), their re-enforcement may be a successful strategy to ameliorate autoimmune diseases including MS.

We therefore investigated the expression and regulatory function of PD-L1 on monocytes in the peripheral blood of healthy subjects and MS patients, and its modulation by the approved MS drug IFN-ß (*study III*). Although there were only low quantities of PD-L1 on unstimulated human monocytes, its expression was strongly upregulated by IFN-ß and led to reduced APC functions *in vitro*. In particular blocking PD-L1 on monocytes markedly increased their capacity to stimulate secretion of cytokines (IL-2 and IFN-γ) by and proliferation of glatiramer acetate- (a random polymer of four amino acids found in myelin basic protein, MBP) or superantigen-activated autologous $CD4^+$ T cells. Monocytes from healthy subjects and untreated patients with MS had similar baseline levels and inducibility of PD-L1 in response to IFN-ß *in vitro*. Moreover, MS patients treated with IFN-ß *in vivo* for 6 months had 8-fold more PD-L1 mRNA transcripts than before treatment (**Figure 2**).

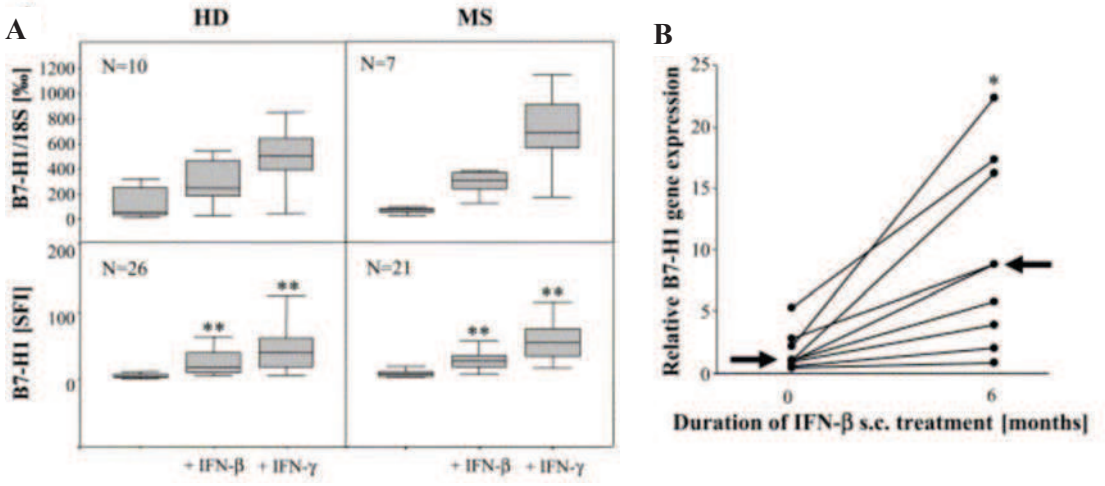

Figure 2. Modulation of PD-L1 (B7-H1) expression by IFN-ß in MS patients *in vitro* and *ex vivo*. (A) PD-L1 mRNA (upper row) or protein (lower row) was measured by qRT-PCR and flow cytometry on monocytes of healthy donors (HD) and in MS patients. Cells were cultured without or in the presence of IFN-ß (1000 U/ml) or IFN-γ (500 U/ml; positive control) for 48 hrs. The box plots show the median, quartiles and minimum-maximum rations of copy numbers of PD-L1 relative to the 18S housekeeping gene (mRNA), or SFI values (protein). ** $p<0.01$ (ANOVA and subsequent post hoc tests). (B) PD-L1 expression was determined by qRT-PCR in PBMC of MS patients before (n=9) and 6 months after (n=9) initiation of IFN-ß1b treatment (8 MIU s.c. every other day). Basal mRNA levels of one donor were set to 1 and the remaining mRNA levels were related to this. Arrows indicate medians (*$p<0.05$, two-sided t-test). **Adapted from (*105*).**

Our results suggested that the anti-inflammatory effect of IFN-ß treatment was partially due to PD-L1 expression. We concluded that therapies that aim to increase PD-L1 expression on monocytes/APCs might be a promising approach to treat MS. On this note, PD-L1 fusion protein therapeutics have been recently tested in experimental models of collagen-induced arthritis and colitis where they appear to have protective effects (*106, 107*).

To study the role of PD-L1 expression on different infiltrating and resident myeloid cells/APCs in regulating local T-cell function in the CNS itself, we took advantage of the relapsing PLP-peptide/SJL and chronic MOG-peptide/C57BL/6 EAE model (reviewed in (*108*); *study IV*). Despite limitations, EAE has proven very useful to examine inflammatory aspects of MS and develop new therapeutic concepts as it shares many immunopathological characteristics with the human disease. Using this model, Latchman and colleagues had previously shown that transfer of wild-type encephalitogenic T cells into PD-L1-deficient recipient mice exacerbated EAE which already hinted to a critical function for PD-L1 in limiting pathogenic effector T cells responses in the target tissue (*109*). Therefore our first aim was to dissect the expression pattern of PD-1 ligands on myeloid APC cell types in the CNS during acute EAE. PD-L1 and PD-L2 were differentially expressed on discrete APC populations in the inflamed CNS with high PD-L1 levels on macrophages, monocyte-derived DCs and microglia. Increased PD-L1 expression in the CNS during EAE was attributable to the infiltration of PD-L1$^+$ myeloid cells and up-regulation on microglia. Our data demonstrated that the majority of CNS CD4$^+$ T cells isolated from the inflamed nervous tissue during acute EAE were PD-1$^+$, and T cells specific for relapse-associated myelin epitopes expressed PD-1 upon antigen stimulation in the CNS. PD-L1 and PD-1 had mainly inhibitory functions on CNS T cells. PD-L1 negatively regulated the stimulation of activated PD-1$^+$ T_H cells in co-cultures with microglia and different CNS-infiltrating APCs presenting endogenously processed peptides. The preponderance of IFN-γ^+ versus IL-17$^+$ T cells in the CNS of PD-L1$^{-/-}$ mice suggested that PD-L1 more selectively suppresses T_H1 than T_H17 responses *in vivo*. In contrast, blockade of PD-L2 had less

pronounced regulatory effects (**Figure 3**). Overall, the results suggested that local PD-L1:PD-1 interactions attenuated autoreactive T cell responses thereby restricting immunopathological damage in the CNS.

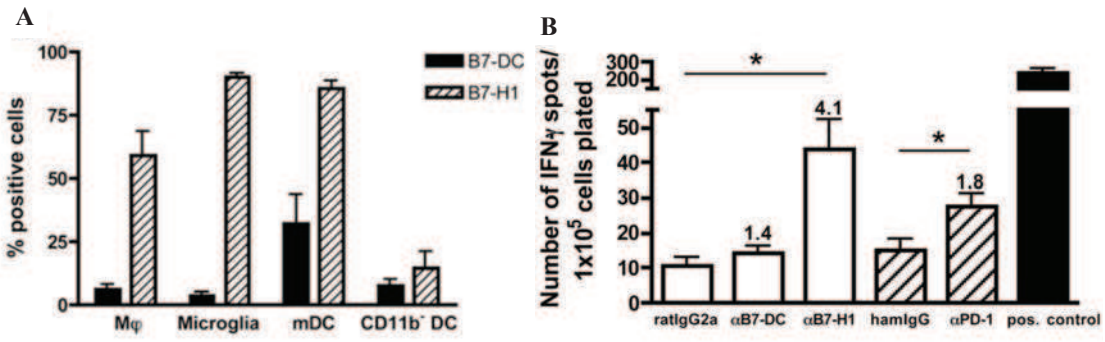

Figure 3. (A) Expression of PD-L1 (B7-H1) and PD-L2 (B7-DC) on microglia and different CNS-infiltrating APC subsets in the inflamed CNS. Cells were isolated from $PLP_{178-191}$-primed mice at the peak acute phase of relapsing EAE (day 14-15 post-immunization) and stained for flow cytometry with PD-L1, PD-L2 and the following markers: Macrophages were $CD45^{high}CD11c^{-}CD11b^{+}$, microglia were $CD45^{low}CD11b^{+}$, monocyte-derived DCs (mDCs) were $CD45^{high}CD11c^{+}CD11b^{+}$ and $CD11b^{-}$DC were $CD45^{high}CD11c^{+}CD11b^{-}$. Data are plotted as mean percentage ±SEM from at least 3 independent experiments. Gates were set respective to isotype controls. (B) The frequency of IFN-γ-producing cells was determined by ELISPOT from the pooled CNS of $PLP_{178-191}$-primed SJL mice (n=5-10) at the peak acute phase of relapsing EAE. Endogenous presentation (i.e. without addition of exogenous PLP-petides) was determined in the presence of blocking antibodies to PD-L1, PD-1 or isotype controls. Data are representative of 4 independent experiments. * $p<0.05$ (unpaired t-test). **Adapted from (*110*).**

Our work confirmed results of a second study (*111*) that identified microglia/macrophages and invading T cells as the main cellular source of inhibitory PD-L1 in the inflamed CNS tissue during EAE. In addition, *in vitro* exposure of human monocytes and microglial cells to T_H1 cell-derived supernatants significantly upregulated PD-L1 mRNA (*111*). In the meantime PD-L1 has also been detected on Iba-1^{+} macrophage/microglia and $GFAP^{+}$ astrocytes mostly in sub-/acute MS lesions but also in normal-appearing white matter of specimens from MS patients (*112, 113*), which emphasizes the therapeutic potential of PD-L1:PD-1 modulation in human neuroinflammatory diseases.

Homeostatic functions of GFAP$^+$ astrocytes

V. **Schreiner B**, Romanelli E, Liberski P, Ingold-Heppner B, Sobottka-Brillout B, Hartwig T, Chandrasekar V, Johannssen H, Zeilhofer HU, Aguzzi A, Heppner F, Kerschensteiner M, Becher B (2015) Astrocyte depletion impairs redox homeostasis and triggers neuronal injury in the adult CNS. **Cell Reports**. doi: 10.1016/j.celrep.2015.07.051. [Epub ahead of print] PMID: 26299968

There is accumulating evidence, that glial cells including astrocytes exert a broad control of CNS tissue homeostasis besides their engagement in cross-talk with CNS-infiltrating T cells (*48*). As discussed in the introductory part above, *in vitro* studies with isolated cells or brain slices have taught us much about basic properties of astrocytes. However, our knowledge of maintenance functions of astrocytes in the adult CNS under physiological conditions *in vivo* is still incomplete (*study V*).

Therefore, we decided to use a model by which tamoxifen-inducible Cre-ERT2 allows temporarily precise postnatal induction of diphtheria toxin-A (DTA) in GFAP$^+$ astrocytes (GFAPCreERT2:DTA model (*114, 115*)). DT is a toxin that, upon entry into the cytoplasm, 'paralyzes' target cells by catalyzing the inactivation of elongation factor 2, resulting in termination of protein synthesis and subsequent cell death (*116*). We confirmed the specificity of this genetic targeting approach by flow cytometry and immunohistochemical analysis of GFAPCreERT2 mice crossed to a Rosa26-tdTomato reporter mouse line (**Figure 4A**). In the spinal cord, the Cre-recombinase was primarily active in the subpopulation of GFAP$^+$ astrocytes whereas only a smaller fraction of astrocytes labeled with the more-abundant marker S100$^+$ were targeted (**Figure 4B**). At day 6 after the start of tamoxifen treatment, the expression of DTA led to significantly reduced tdTomato$^+$ cell numbers compared to controls (**Figures 4B and C**). This suggested that a substantial fraction of GFAP$^+$ astrocytes, in which the Cre recombinase was active, were dying or arrested in an inanimate state with blocked (reporter) protein synthesis.

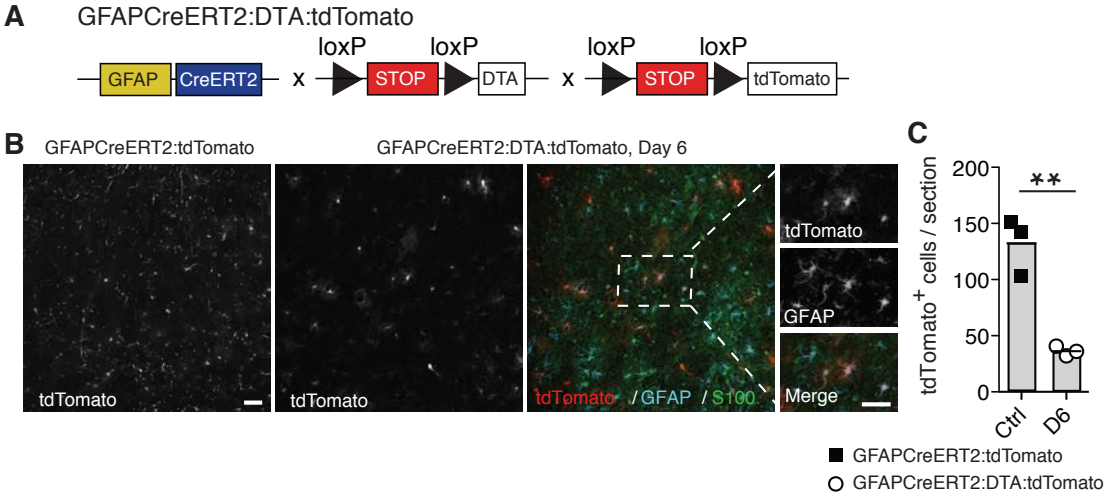

Figure 4. (A) Schematic diagram of GFAPCreERT2 × DTA (GFAPCreERT2:DTA) mice, crossed with Rosa26-tdTomato transgenic reporter mice (Ai14) to label cells in which Cre-mediated recombination takes place. Tamoxifen i.p. injections were performed at days 1-5. (B and C) Spinal cord samples were immunostained for GFAP and S100, and the numbers of tdTomato$^+$ cells as well as their co-localization with the astrocyte marker proteins were quantified. (B) Representative images of tdTomato$^+$ cells in sections from control GFAPCreERT2:tdTomato mice and mice in which the DTA expression is additionally activated (GFAPCreERT2:DTA:tdTomato) are shown. The insets show tdTomato$^+$ cells with astrocytic morphology and co-localizing with GFAP. The number of tdTomato$^+$ astrocytes per section were significantly reduced at day 6 in GFAPCreERT2:DTA:tdTomato-D6 compared to GFAPCreERT2:tdTomato-Ctrl mice. For quantifications, see (C). In (C), squares and circles represent individual mice and the bar the mean value (n = 3/group; two-tailed Student's t test). The scale bars represent 200 µm (B) and 50 µm (inset in B)**. Adapted from (*117*).**

As the most-pronounced pathology in tamoxifen-treated GFAPCreERT2:DTA mice was found in the cervical spinal cord, our subsequent analysis focused on this region. i.p. injections of tamoxifen in GFAPCreERT2:DTA mice resulted in a rapid and severe paresis that started asymmetrically in both upper limbs, later progressed to all four extremities (onset at days 5-8), and was accompanied by weight loss (**Figure 5A**). In the spinal cord, we found that the numbers of neurons and oligodendrocytes were reduced at day 6, paralleling the appearance of the clinical phenotype. In contrast, a comparable number of S100$^+$ astrocytes (of which only few express the Cre recombinase and the loss of which is therefore likely obscured by a gliotic response) was present at all time points, suggesting that an overall astroglial support structure persisted (**Figures 5B and C**). Moreover, neuronal injury occurred in the absence of any major microvascular damage and microbleeds or leakage of toxic blood products.

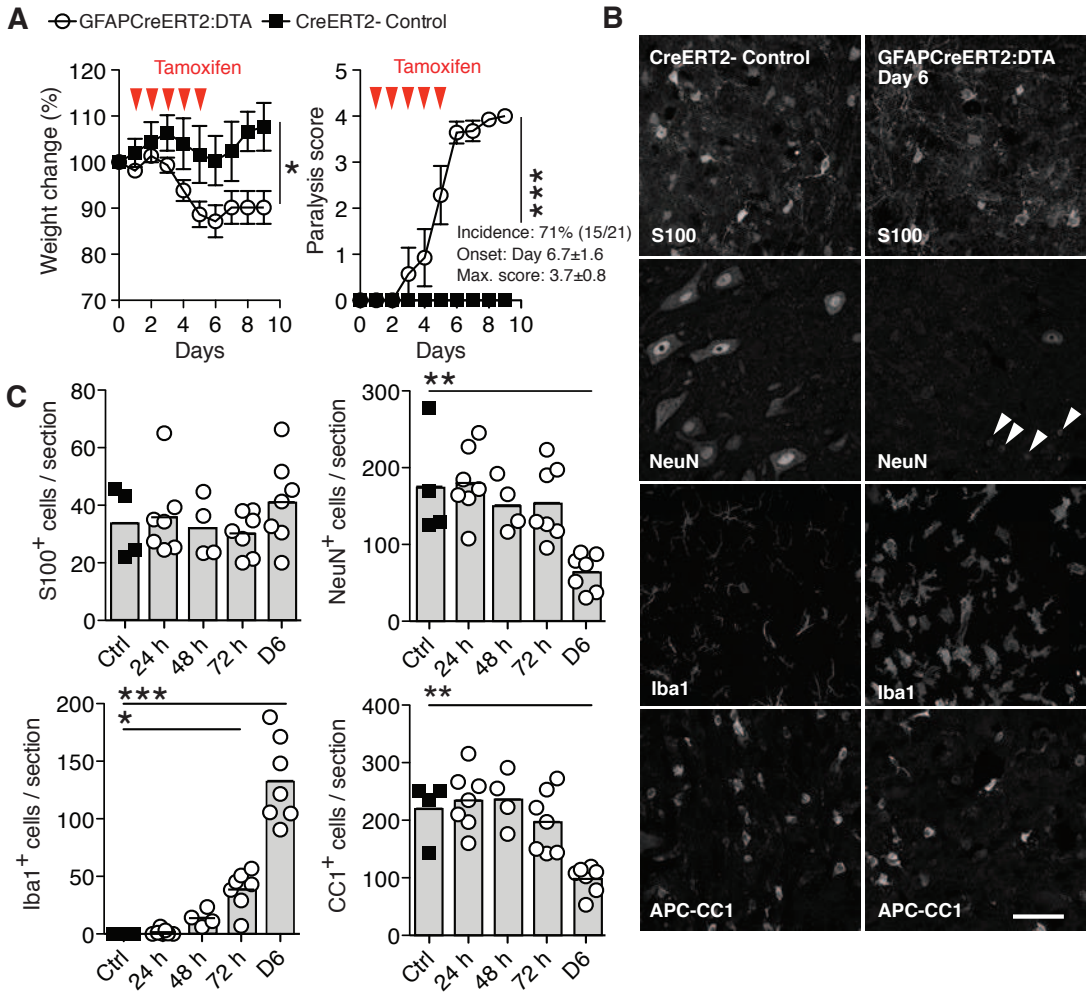

Figure 5. Paralysis of Astrocytes in GFAPCreERT2:DTA Mice leads to a rapid and severe neuronal motor phenotype. (A-C) GFAPCreERT2:DTA mice were i.p. injected with tamoxifen at days 1–5. (A) At days 5–8, mice exhibited a severe paralysis that rapidly progressed to all four limbs and was accompanied by weight loss (mean ± SEM; n = 3-7/group; two-way ANOVA test). (B and C) Cervical sections were immunostained at the indicated time points to assess remaining astrocyte structural integrity (S100) and to detect neurons (NeuN), oligodendrocytes (APC-CC1), and microglia (Iba1). Arrows in (B) indicate neurons undergoing cell death. For quantifications, see (C). Values of individual mice are shown by circles (GFAPCreERT2:DTA; n = 4-7 mice/time point) and squares (CreERT2$^-$ control tissue; n = 4). Group means are indicated by bars. Statistical analyses were performed via one-way ANOVA and Dunnett's multiple comparison test versus Ctrl. ⁎p < 0.05; ⁎⁎p < 0.01; ⁎⁎⁎p < 0.001.The scale bar represents 50 μm (B)**. Adapted from (*117*).**

Given the observed morphological activation and proliferation of microglia (**Figures 5B and C**), we examined whether microglial-derived factors contributed to neuronal demise and the functional deficits in GFAPCreERT2:DTA mice. To directly assess the role of microglia in astrocyte-driven motoneuron injury in our model, we used the colony-stimulating factor 1 receptor (CSF1R) antagonist PLX5622, which has been shown to efficiently eliminate microglia from the adult brain (*118*). CSFR1 blockade decreased microglial content as assessed by the number of Iba1$^+$

cells in cervical sections (**Figures 6A and B**). However, at the same time point, the loss in motoneuron numbers (**Figures 6A and B**) and the severity of clinical paralysis (**Figure 6C**) was not significantly different from mice fed with control chow. These findings indicated that, in our model, microglia are rather bystanders and their activation had no impact on neuronal loss and clinical decline.

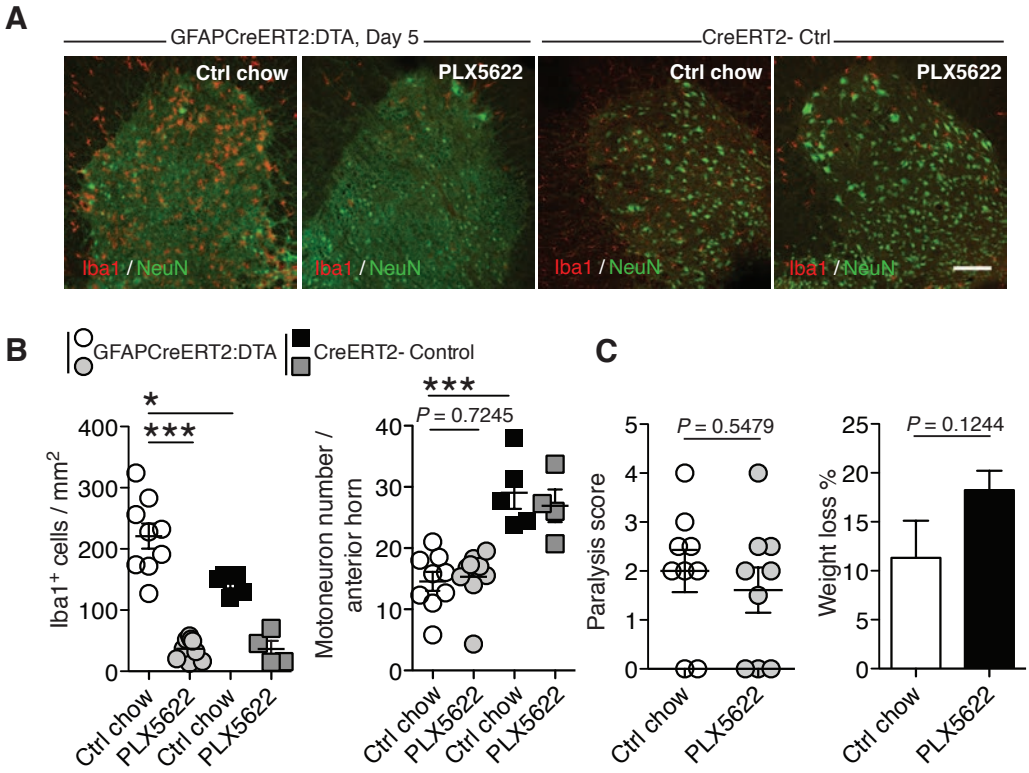

Figure 6. Motoneuron loss in spinal cords of GFAPCreERT2:DTA mice is independent of microglial bystander activation. GFAPCreERT2:DTA (n = 9/group) and CreERT2-control mice (n = 4 to 5/group) were pre-/treated with the oral CSF1R inhibitor PLX5622 (1.2 g/kg chow) or vehicle for 1 week to eliminate microglia and kept on this diet throughout the experiment. (A) Representative Iba1 and NeuN immunofluorescent stainings from the anterior horn region at day 5 of tamoxifen i.p. application showing loss of moto-/neurons despite robust decreases in microglial numbers. (B) Quantifications of Iba1+ and motoneuron cell bodies. (C) There was no statistical difference in paralysis scores at day 5 between PLX5622- and vehicle-treated GFAPCreERT2:DTA mice. Mice were pooled from two independent experiments, and asterisks indicate significance by unpaired Student's t test; ∗p < 0.05; ∗∗∗p < 0.001. Error bars represent mean ± SEM. The scale bars represent 100 µm (A). **Adapted from (*117*).**

Although the acute inhibition of astrocyte protein synthesis might result in the lack of trophic support for neurons, we reasoned that it was equally likely that it led to inactivation of critical glial-detoxifying pathways. Mutations in superoxide dismutase 1 (SOD1) cause a rare form of fALS, and studies using transgenic rodents expressing mutant human SOD1 have indeed demonstrated that motoneurons are particularly vulnerable to toxic activity mediated by astrocytes (*119, 120*). In addition, previous co-culture

experiments suggested that neurons depend on the antioxidant potential of astrocytes for their own defense against oxidative stress *in vitro* (*121*). To assess the redox-scavenging capacity in the $GFAP^+$ astro-/glial cell pool in our *in vivo* model, we measured the mRNA and protein expression of different ROS-detoxifying enzymes. We detected diminished ROS-scavenging capabilities in the glial pool in GFAPCreERT2:DTA mice which were accompanied by increased superoxide levels and enhanced oxidative DNA damage in the spinal cord tissue. Reactive oxygen and nitrogen species (ROS/RNS) scavenger treatment of mice (*122*) significantly increased the number of surviving neurons including a preservation of motoneurons. In addition, we measured serum concentrations of neuron-specific enolase (NSE), which is systemically released upon neuronal injury. NSE serum levels in GFAPCreERT2:DTA mice were significantly increased compared to $CreERT2^-$ controls with mean NSE levels lying between values reported for models of ischemic stroke and inflammatory CNS insults (*123*). Again treatment with ROS/RNS scavengers reduced serum NSE levels significantly. ROS/RNS scavenging not only improved neuronal survival but also prevented clinical deficits (at days 5 or 6).

Collectively, our results emphasized the magnitude to which neuronal viability depends on the functional integrity of astrocytes in the adult CNS. They suggested that the subpopulation of $GFAP^+$ astrocytes maintained neuronal health by controlling redox homeostasis in the adult CNS. This could have implications not only for neurodegenerative diseases like ALS, but also for neuroprotective interventions, that might be more likely to fail if they do not also target the glial, metabolically active environment of neurons.

CONCLUSIONS AND OUTLOOK

It is becoming apparent that the CNS environment allows immune responses within its borders to fight tumors and pathogens but local control processes involving resident glial cells are critical to protect vulnerable nervous tissue. In addition, it is increasingly appreciated that glial cells in particular astrocytes have diverse maintenance functions both in the inflamed CNS but also under non-pathological conditions that reach far beyond pure scar formation and structural support. An improved understanding of mechanisms of glial immune regulation and homeostatic functions is a key requirement for the advancement of therapeutic neuroprotective strategies. Here, we uncover the PD-L1:PD-1 pathway as an important component of the local immune signaling networks that restrict immune responses, and identify the redox scavenging capabilities of the astroglial cell pool as a critical factor for maintaining neuronal health in the adult CNS.

We have learnt during our studies, that there is a considerable heterogeneity in the population of astroglia. Distinct astrocyte subsets may differ in their impact on the pathogenesis of MS during different disease stages. In the future, we therefore attempt to further characterize astroglial cell phenotypes and glial-derived molecules that contribute to the immunopathology of inflammatory CNS diseases such as MS.

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